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High-fat diet or low-protein diet changes peritoneal macrophages function in mice

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Abstract

Background: Obesity and protein malnutrition are major food problems nowadays, affecting billions of people around the world. The nutrition transition that has occurred in recent decades is changing the nutritional profile, reducing malnutrition and increasing the percentage of obese people. The innate immune response is greatly influenced by diet, with significant changes in both malnutrition and obesity. Therefore, we investigate the effects of protein malnutrition and obesity in nutritional and immunological parameters in mice.

Results: Peritoneal macrophages of malnourished animals showed reduced functions of adhesion, spreading, and fungicidal activity; production of hydrogen peroxide and nitric oxide were lower, reflecting changes in the innate immune response. However, the high-fat animals had macrophage functions slightly increased.

Conclusions: Animals subjected to low-protein diet have immunosuppression, and animals subjected to high-fat diet increased visceral adipose tissue and the presence of an inflammatory process with increased peritoneal macrophage activity and similar systemic changes to metabolic syndrome.

Keywords: Obesity, Malnutrition, Nutritional transition, Immune response, Macrophages

Background

A few decades ago, it was considered unlikely a pandemic of obesity, but from the 1970s, the number of obese people has increased to become worrisome in the 1990s [1]. FAO and WHO data [2, 3] also show that the protein malnutrition was a major nutritional disorder throughout the world. However, there has been a rapid change in nutritional status of the population; it characterizes the nutritional transition [4, 5]. Changes in diet can lead to significant changes in metabolism, such as the development of various diseases due to the lack of quality in the immune response.

Data from our laboratory and other authors show that among the alterations in malnutrition are the changes in hematopoietic tissue with modification in extracellular matrix components [6, 7]. The literature describes alterations in the cell cycle of hematopoietic progenitor [8], reducing the production of cells and hematopoietic

precursors [6], both in the erythrocyte series, as in the leukocytes series, leading to anemia and leukopenia [9–11]. Protein malnutrition alters both specific and nonspecific immune response of the individual, against infectious agents [11, 12].

Obesity is associated with increased levels of leptin and adipocytes, the latter being the main producer of leptin, as well as other mediators, mainly inflammatory, such as tumor necrosis factor alpha (TNF α), IL-1 β , and IL-6 that may act directly or indirectly in hematopoietic cells [13]. Leptin also acts on the immune system [14], stimulating the production of white blood cells (WBC) in the bone marrow and their migration. It also increases the production of pro-inflammatory cytokines such as TNF α , IL-6, and IL-12, macrophage adhesion and phagocytosis and stimulates the proliferation of T cells, leading to increased immune competence [15]. Recent studies have shown that obesity leads to decreased blood flow in adipose tissue, causing a hypoxia, which initiates a pro-inflammatory process [16, 17], with increased production of acute phase proteins such as C-reactive protein [18] and cytokines such as TNF α and IL-6 [19]. The production of pro-inflammatory cytokines commonly

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observed in situations of obesity may have effects on hematopoiesis, inducing or inhibiting the proliferation and differentiation of cells present in bone marrow. Inflammatory mediators also have the ability to activate other cells to produce different mediators that perhaps might influence hematopoietic complex mechanism types [16, 17]. Some effects exert proliferation (such as GM-CSF), while others have apparently hematopoietic inhibitory effect on proliferation (TNF α and IFN γ , for example) [20, 21].

Previous work from our group showed some similarities in immunological parameters between the metabolic condition of malnourished and high-fat fed. We found decreased peritoneal, bone marrow, and serum leukocyte cellularity, as well as reduced production of TNF α and activation of transcription factor NF- κ B both in the low-protein diet [10, 22] and in the high-fat diet [23].

The aim of this work is to investigate the similarities and differences between these two diets in macrophage function.

Methods

Animals and treatments

Swiss Webster mice, males, 2–3 months old, were obtained in the Faculty of Pharmaceutical Sciences, University of São Paulo. The research project was approved by the Ethics Committee on the Use of Animals of University of São Paulo (protocol: CEUA/FCF/366). The control, low-protein, and high-fat diets (Table 1) were prepared in our laboratory and stored at -4°C until the use. Control diet was prepared for adult mice, according to the recommendations of the American Institute of Nutrition (AIN-93M) [23, 24]. The low-protein diet has 2 % of protein [22]. The high-fat diet has 30 % fat in the form of lard, and all other ingredients have been

increased by 33 % to provide a balanced nutrient proportion compared to control because, in a pilot project, we found that feed intake in animals subjected in the fat diet was lower than that in the control on this ratio [25]. The animals were housed individually in cages under similar environmental conditions, with clear light/dark cycle of 12/12 h, temperature of $22 \pm 2^{\circ}\text{C}$, and relative humidity of $55 \pm 10\%$. The adjustment period to cages was 10–15 days (until the body weight had stabilized). In this period, the animals received the control diet and water ad libitum and were weighed every 48 h to evaluate body weight. After the adaptation, the animals were randomly separated into three groups: control (C), malnourished (M), and high-fat (H). The control group continued to receive the control diet while malnourished began receiving low-protein diet and high-fat group receiving the high-fat diet. During the experiment, body weight and feed were evaluated every 48 h. At the end of 5 weeks, the animals were euthanized for collection of biological material.

Measurement of visceral fat and Lee index

Subsequent euthanasia, the retroperitoneal and periepididymal adipose pads were dissected and immediately weighed. We carry the body mass index (BMI) by Lee index [26], where we use the following link: $(\text{weight (g}^{0.33}) / \text{naso-anal measured (mm)})$. These values may characterize the change in mass of mice after the induction of obesity.

Phenotypic characterization of peritoneal cells

Phenotypic characterization of peritoneal lavage was performed by flow cytometry. Aliquots of 1×10^5 cells/ml of cell suspension from the sample of peritoneal lavage cells were suspended in RPMI 1640 medium (Cultilab[®], Brazil), pH 7.4. Antibodies were added and incubated for 30 min with 2 μL antibody APC-F4/80 (Cat 113006, Lot#b146514 Biolegend[®]) and/or 2 μL FITC-CD11b antibody (Cat 11-0112-82, Lot#E033743 e Bioscience[®]), stirred, protected from light. The acquisition was made on FACSCanto II (Becton Dickson[®], USA) acquiring 10,000 events analyzed and compensated by software FLOW JO 7.6 (TreeStar[®], USA).

Cell culture

Peritoneal cells were obtained by washing the peritoneal cavity with 5 mL of sterile, pyrogen-free McCoy's 5A medium (pH 7.4) supplemented with 10 % fetal bovine serum, glutamine 2 mmol/L, 100 U/mL penicillin, and 100 mg/mL streptomycin (Cultilab, Brazil). Cells were spun down (1500 rpm for 10 min at 4°C) and resuspended twice in McCoy's 5A culture medium. Cell viability was determined by trypan blue exclusion. Cultures rich in macrophages were obtained by incubating 1×10^6 cells/mL in 24- or 96-well polystyrene culture plates for 2 h at 37°C in a 5 % CO_2 -humidified air environment.

Table 1 Composition of experimental diets

Ingredients (g/kg)	Control diet ^a	Malnourished diet	High-fat diet ^b
Cornstarch	640	740	193.38
Sucrose	100	100	133.56
Casein	120	20	186.98
Corn oil	80	80	53.42
Lard	–	–	300
Cellulose	50	50	66.78
Mineral mix	35	35	46.74
Vitamin mix	10	10	13.36
Methionin	1.8	1.8	2.4
Choline bitartrate	2.5	2.5	3.34
Tert-butylhydroquinone	0.01	0.01	0.04

^aAccording to AIN-93M

^bIngredients have been increased by 33 % to provide a balanced nutrient because that feed intake in animals that subjected the high-fat diet was lower than that in the control on this ratio [25]

Non-adherent cells were removed by vigorous washing three times with McCoy's 5A medium. Macrophages were incubated with or without LPS 1 mg/mL (*Escherichia coli*, serotype 055:B5, Sigma Chemical Company). After 2 or 24 h of incubation with or without LPS, the supernatant was used to determine adhesion, spreading, phagocytosis, and killing, and hydrogen peroxide (H_2O_2) is an oxide nitric (NO) concentrations in the culture supernatant. The entire procedure was executed under aseptic conditions, and all materials used were sterile and free of pyrogen.

Evaluation of cell adhesion ability of peritoneal cavity

Cells in peritoneal cavity were plated in a 96-well plate at a concentration of $1 \times 10^5/150$ uL in McCoy's 5A culture medium and incubated for 1 h at 37 °C, 5 % CO_2 , in a humidified atmosphere. Thereafter, washing was performed with culture medium to remove non-adherent cells. The adhered cells were cultured in the presence or absence of 1.25 μ L/mL of LPS (serotype O55:B5 Sigma-Aldrich®) for 2 to 24 h at 37 °C, 5 % CO_2 , in a humidified atmosphere. The number of adherent cells was assessed by crystal violet method. The wells were washed with PBS pH 7.4, and the cells fixed with 30 μ L of 4 % paraformaldehyde for 10 min at room temperature. After fixation, 50 μ L of crystal violet in methanol solution (0.25 g of crystal violet; PA 10 mL methanol; 40 mL of deionized water) was added to each well. The plate was kept in the dark for 30 min at room temperature. After washing the wells with PBS pH 7.4, 50 μ L of 0.1 M sodium citrate pH 4.2 was added. The supernatant was transferred to another plate, and its absorbance determined at a wavelength of 550 nm. The results were expressed as absorbance/ 1×10^5 cells.

Spreading tests on peritoneal cells

Using 24-well culture plates (Falcon, 3047, Becton-Dickinson), 13–18-mm sterile coverslips were placed on the bottom of each well. Macrophage-rich cultures were obtained by 1-h incubation (37 °C, 5 % CO_2) of 1×10^6 cells/mL in 24-well plates with coverslips. Non-adherent cells were removed by vigorous washing three times with McCoy's 5A sterile medium (Sigma Chemical Company, USA), pH 7.4. Adherent cells were then cultured in 1 mL of McCoy's 5A sterile medium, pH 7.4, in the presence or absence of 1.25 μ g/mL of LPS (*E. coli*—B5:055, Sigma, Chemical Company, USA). The plates were incubated for 2, 24, 48, and 72 h at 37 °C, under a 5 % CO_2 atmosphere. After this period, the supernatants were collected and the coverslips were washed with PBS Dulbecco, pH 7.4, and fixed for 20 min with 2.5 % glutaraldehyde (Sigma Chemical Company, USA) and stained by May-Grunwald-Giemsa solution. Using optical microscopy the adhered cells were distinguished from those which were spread.

Phagocytic and fungicidal activity of peritoneal macrophages

A suspension of *C. albicans* ATCC Y-837 obtained from a 12-h culture in Sabouraud agar (Difco®) was opsonized with homologous serum obtained from normal mice. Yeast cells were counted in a Neubauer chamber and viability was evaluated using 1 % methylene blue. Only yeast suspensions with over 95 % viability were used. The suspensions were adjusted to 2×10^7 yeast cells/mL. In sterile plastic tubes, 500 μ L (2×10^6 /mL) of the peritoneal cells suspension and 500 μ L of the opsonized *C. albicans* solution were added maintaining a proportion of 1 cell is to 10 yeast cells. The tubes were incubated at 37 °C, under agitation at 10 rpm, and aliquots were taken after 30, 60, 90, and 120 min and were cytocentrifuged (Incibras®, Brazil). The coverslips were immediately fixed and stained by May-Grunwald-Giemsa solution. The control of the reaction consisted of tubes containing 500 μ L of PBS Dulbecco and 500 μ L of the peritoneal cells suspension. For the evaluation of phagocytosis, at least 200 peritoneal cells were counted and those which presented one or more internalized *C. albicans* cells were considered as having phagocytic activity, the values being expressed in percentage. The fungicidal activity was evaluated accordingly to the technique described by Herscowitz [27] and modified by us.

In this technique, live yeast cells are colored blue by the May-Grunwald-Giemsa stain while the dead yeast cells are not colored at all. The number of yeast cells phagocytosed is variable from cell to cell so the fungicidal activity was expressed by scoring. When the macrophages presented one *C. albicans* dead, we multiplied by one (score 1), two *C. albicans* dead multiplied by two (score 2), three *C. albicans* dead multiplied by three (score 3) and more than four *C. albicans* dead multiplied by four (score 4). The fungicidal activity was evaluated counting at least 200 macrophages that had phagocytosed *C. albicans* multiplied for the score according the classification. All samples were processed in duplicate.

NO and H_2O_2 determination in culture supernatants

Nitric oxide (NO) production was determined according to the Griess colorimetric method [28]. The determination of hydrogen peroxide (H_2O_2) was performed by the method of peroxidase-dependent oxidation of phenol red adapted to micro-assay for PICK & MIZEL [29].

Statistical analysis

Results were checked for distribution normality and homoscedasticity. Means from the C, M, and H groups were compared by unpaired Student's *t* test or an equivalent non-parametric test (Mann-Whitney). The results from the assays using peritoneal macrophages were analyzed with one-way analysis of variance, Tukey post

test, or an equivalent non-parametric test (Kruskal-Wallis, Dunn post hoc). Statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software, Inc., USA), and the level of significance adopted was 0.05.

Results

Food intake and weight gain

Feed intake was similar between the control and malnourished; however, the consumption of high-fat group was 33 % lower, as already shown in previous experiments in our laboratory. Nevertheless, the calorie intake was similar in all groups because the high-fat group has a higher proportion of lipids in the diet (Table 2).

In the beginning of the experiment all groups had similar body weight and consumed similar amount of calories for the entire period, the composition of the feed, by varying the amount of lipids and proteins, led high fat group to increase their weight and malnourished animals to significantly decrease body weight.

The animals subjected to high-fat diet had a higher Lee index in comparison to the control group and the malnourished group ($p < 0.05$). The total weight of the fat pads showed significant differences in the HL group compared to other groups, especially the epididymal fat (Table 2). However, malnourished and control groups showed no significant differences.

Phenotypic characterization of cells in the peritoneal cavity

Phenotypic evaluation of the peritoneal cavity population was done by flow cytometry using the CD11b (FITC) and F4/80 (APC). The double-labeled cells were classified as macrophages. The proportion of macrophages

was shown to be decreased in malnourished animals and similar between control and high-fat animals (Fig. 1).

Adhesion and spreading of peritoneal macrophages

The peritoneal macrophages were cultured for 2 h, and after this period, it was shown that cells of malnourished animals had a reduction in macrophage adherence, whereas cells of high-fat animals had increased adhesion. In addition, the cells responded to all three groups were stimulated with LPS. However, cells cultured for 24 h decreased the response to stimulation by LPS. Malnourished animals continue with reduced adhesion, and the control group and high-fat have similar adhesion capacity (Table 3).

The high-fat group presented a lower amount of sprawling cells after 2 h, at baseline and in the group stimulated with LPS compared to control group. The malnourished group showed even less sprawling cells that high-fat even after the stimulus with LPS. Table 3 shows the reaction to the stimulus, demonstrating that the control and high-fat groups respond to the stimulus, and the cells try to spread in larger quantities. The malnourished group did not respond to LPS stimulation. We also observed the spreading (Fig. 2) capacity after 24 h, where we observe that the cells are much spreading, however, remained high-fat and malnourished groups with a lower percentage of control. Analyzing the response to LPS stimulation, we note that the control and high-fat groups continue to react to the stimulus and the malnourished group showed no significant changes.

Phagocytic and fungicidal activity of peritoneal macrophages

The high-fat group showed an increase of phagocytosis of *C. albicans* after 120 min. However, the malnourished group showed a decrease in phagocytic activity after 90 min, as shown in Table 4.

After the analysis of the opsonized and non-opsonized groups, comparing the control, malnourished, and high-fat groups, we note that at 30, 60, and 90 min, the malnourished group had reduced phagocytosis, and the control and high-fat groups were not significantly different. However, at 120 min, there is a similarity in the phagocytic activity between the three groups analyzed.

The fungicidal activity (Fig. 3) was shown to be decreased in the peritoneal cells of malnourished animals at all times evaluated, while it increased in animal cells submitted to high fat diet after 90 min of assay.

Evaluation of hydrogen peroxide and nitric oxide production by peritoneal macrophages

The malnourished group did not react to the stimulus with PMA. Although the groups did not present similar

Table 2 Food intake, weight gain, fat pads, and lipid profile

Groups	Control	Malnourished	High-fat
Food intake (g/day)	7.0 ± 0.8	7.1 ± 0.5	4.9 ± 0.8 ^a
Calorie intake (Kcal/day)	29.6 ± 2.1	29.9 ± 3.4	29.1 ± 3.3
Lipids intake (g/day)	0.6 ± 0.1	0.6 ± 0.1	1.9 ± 0.2 ^a
Protein intake (g/day)	0.9 ± 0.1	0.2 ± 0.1 ^a	0.9 ± 0.1
Initial body weight (g)	43.4 ± 0.8	44.5 ± 0.9	43.7 ± 0.9
Final body weight (g)	44.6 ± 0.6	38.5 ± 0.7 ^b	50.5 ± 1.0 ^a
Weight gain (%)	6.1 ± 5.0	-16.7 ± 5.5 ^b	17.5 ± 10.9 ^a
Fat pads weight (g):			
Peri-epididymal	2.0 ± 0.3	1.4 ± 0.1 ^b	2.6 ± 0.7
Retroperitoneal	0.5 ± 0.2	0.3 ± 0.1	0.8 ± 0.4
Total	2.4 ± 0.6	1.7 ± 0.4 ^b	3.1 ± 0.8 ^a
Lee index	3.2 ± 0.1	3.0 ± 0.1	3.7 ± 0.1 ^a

Values are presented as mean and SD ($n = 26/\text{group}$)

^aIndicates when there was significant difference between the high-fat and control groups ($p < 0.05$)

^bIndicates when there was significant difference between the malnourished and control groups ($p < 0.05$)

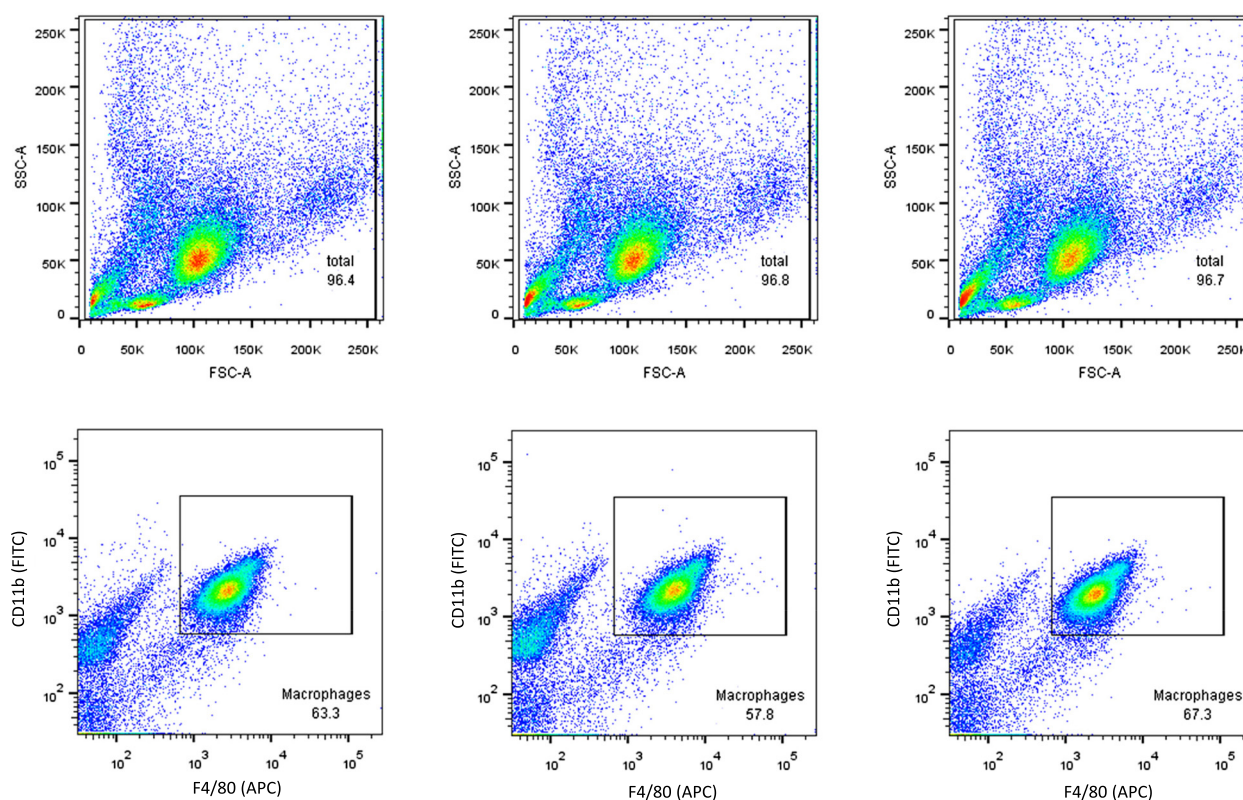


Fig. 1 Representative results of flow cytometry to cellularity of the peritoneal animal of group (a) control, (b) malnourished, and (c) high-fat; double labeled (CD11b+ and F4/80+) group (d) control, (e) malnourished and (f) high-fat

stimulated production of hydrogen peroxide, Table 5 shows that the groups have stimulated representative differences and reduced production in malnourished and higher in high-fat group. In unstimulated conditions cells from all groups did not show statistical difference, but when they are stimulated with PMA cells from the control and high-fat group, present increased production of hydrogen peroxide when compared to the unstimulated.

The control and high-fat groups responded to LPS stimulation and produced more nitric oxide than unstimulated. The malnourished group did not react to the LPS stimulus. In unstimulated conditions, the cells of

animals of all groups showed no statistical difference between them. Although the groups did not present similar stimulated nitric oxide production, Table 5 clearly shows that the groups have stimulated representative differences and greatly reduced production in malnourished and higher in group high-fat group.

Discussion

Changes in dietary components can influence important defense mechanisms against pathogens. We compared the effect of low-protein diet and high-fat diet, on some aspects of innate immunity in mice.

Table 3 Adherence and spreading of peritoneal macrophages

Time (h)	Control	Malnourished	High fat	Control +LPS	Malnourished +LPS	High fat +LPS
Adherence (Abs/1 × 10 ⁵ cel)						
2	0.09 ± 0.01	0.08 ± 0.02	0.12 ± 0.01 ^a	0.11 ± 0.02	0.09 ± 0.02	0.14 ± 0.01 ^a
24	0.11 ± 0.02	0.07 ± 0.01 ^b	0.14 ± 0.03 ^a	0.13 ± 0.03	0.08 ± 0.01 ^b	0.15 ± 0.04 ^a
Spreading (%)						
2	8.4 ± 0.6	3.2 ± 0.1 ^b	3.8 ± 0.4 ^a	91.2 ± 0.8	50.6 ± 0.5 ^b	72.1 ± 3.4 ^a
24	10.2 ± 0.5	3.4 ± 0.3 ^b	7.1 ± 0.4 ^a	98.7 ± 1.1	51.3 ± 1.2 ^b	85.2 ± 2.1 ^a

Results represent means ± SD of the adherence and percentage of spread cells of peritoneal exudate macrophages of control, malnourished, and high-fat animals (n = 18/group)

^aIndicates when there was significant difference between the high-fat and control groups (p < 0.05)

^bIndicates when there was significant difference between the malnourished and control groups (p < 0.05)

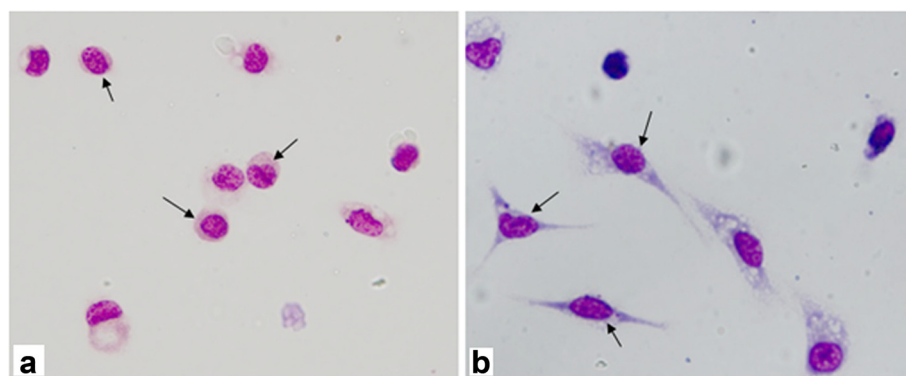


Fig. 2 Representative photomicrograph showing resident peritoneal macrophages (a) and activated spreading (b) (increase $\times 1000$). Stain: May-Grunwald modified Rosenfeld, 1947

The animals subjected to high-fat diet showed an increased body weight and macrophage activity with a pro-inflammatory state, similar to metabolic syndrome. However, animals subjected to low-protein diet had a weight reduction with impaired macrophage immune response. Recent evidence suggests that the feed rich in saturated fatty acids induces an inflammatory process in specific regions of the hypothalamus which promotes negative cross-talk with the signaling pathways of leptin and insulin. As a result, it develops a central state of resistance to the action of these hormones, damaging its anorectic effect [30, 31].

In this work, it was found that the average feed intake was lower in the group that consumed HFD compared to the control groups. On the other hand, due to the energy density of the HFD be higher than the control diet, caloric intake did not differ between the three groups. Importantly, while consumption of the groups have been isocaloric, we noted the effect of HFD feed on several parameters, such as adiposity, resistance to action of insulin, and the inflammatory response of peritoneal macrophages. This reinforces that the diet composition has a direct influence on health, regardless of energy consumption. Extrapolation of these data to humans suggests that strategies to reduce the risk of obesity and metabolic syndrome cannot be limited to the restriction

of calorie intake and should also cover the quality of the diet [23].

The adhesion of macrophages was impaired by malnutrition. The literature reports that the decrease of specific proteins, like fibronectin [32] and integrin, can be the cause of this decrease. The adhesion of macrophages, identified in high-fat animals, can be explained by the action of leptin stimulates adhesion [15] in addition to the production of inflammatory cytokines that stimulate macrophage activity [33]. Works with malnourished humans show decreased adhesiveness due to changes in the activity of macrophages [34].

Few studies have been done with regard to adhesion and spreading of macrophages in malnourished and obese mice. Some studies of our group showed that in malnourished animals, there is a reduction in the spreading of macrophages [12, 35]. In our study, we observed a decrease in the spreading of macrophages in malnourished animals, both in time of 2 h for 24 h. It was also observed that stimulation with LPS had no effect on any of the times evaluated in cells derived from malnourished animals. This reduction in activity may be linked to the impairment of macrophage activity, characterized by decreased production of cytokines stimulating the activation of macrophages, such as IL-6 and TNF [10, 16].

Table 4 Phagocytic and fungicidal activity of peritoneal macrophages

Time (min)	Phagocytosis (%)			Fungicidal activity (score)		
	Control	Malnourished	High fat	Control	Malnourished	High fat
30	9.7 \pm 2.8	10.8 \pm 2.2	10.5 \pm 1.2	28.2 \pm 4.1	16.1 \pm 3.0 ^b	33.7 \pm 5.9
60	43.8 \pm 10.7	42.3 \pm 7.3	43.3 \pm 7.1	73.7 \pm 7.5	40.7 \pm 4.3 ^b	77.1 \pm 4.8
90	48.7 \pm 6.7	39.8 \pm 5.0 ^b	52.8 \pm 7.0	85.8 \pm 4.1	45.7 \pm 2.4 ^b	91.1 \pm 3.1 ^a
120	50.4 \pm 4.0	48.4 \pm 4.0	68.8 \pm 14.1 ^a	93.0 \pm 4.0	60.8 \pm 9.4 ^b	96.5 \pm 2.4

Results represent means \pm SD of the percentage of phagocytic and fungicidal activity of peritoneal exsudate macrophages of control, malnourished and high-fat animals ($n = 15/\text{group}$)

^aIndicates when there was significant difference between the high-fat and control groups ($p < 0.05$)

^bIndicates when there was significant difference between the malnourished and control groups ($p < 0.05$)

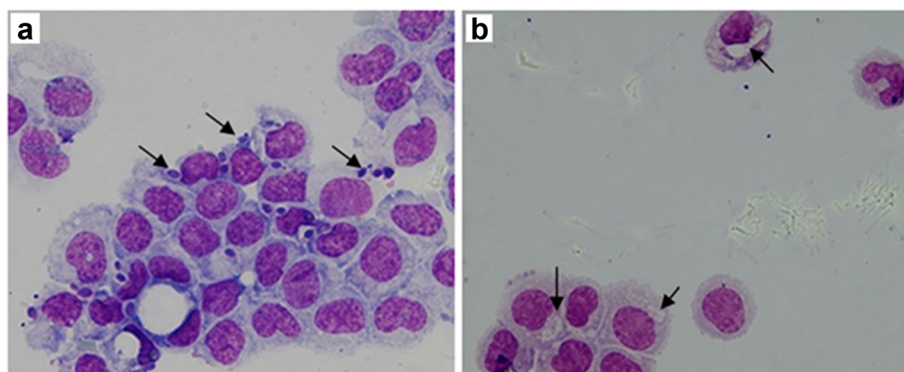


Fig. 3 Representative photomicrograph showing the fungi *Candida albicans* phagocytosis by peritoneal macrophages. **a** The living fungi stained basophilic mode (arrows). **b** Phagocytosed and killed fungi (arrows), which are not stained (increase $\times 1000$). Stain: May-Grunwald modified Rosenfeld, 1947

Phagocytosis is a process of particle aggregation in excess of $1\ \mu\text{m}$. Its main objective is death and/or inactivation of pathogens [36]. The decrease in phagocytosis of cells in malnourished animals can be related to the decreased production of pro-inflammatory cytokines that stimulate macrophage activation. The increase phagocytosis in cells derived from animals in the high-fat group may be due to the amplification of the production of pro-inflammatory cytokines that stimulate macrophage activation [37]. The phagocytic processes are driven by a finely controlled rearrangement of the cytoskeleton. A variety of signals may converge to rearrange the actin cytoskeleton in a phagosome. Studies have shown the complexity of the phagocytic signals, such as the involvement of lipid complexes and signaling in the transduction of signals from the cytoskeleton phagocytic receptors [38, 39].

The fungicidal activity is decreased in the malnourished group. This may be due to decreased production of reactive species of oxygen and nitrogen, essential for this process [40, 41]. The high-fat group showed an increase in fungicidal activity. As we have increased the production of reactive species of oxygen and nitrogen in high-fat animals, this can enhance the fungicide process.

Nitric oxide (NO) is a basic nitrogen reactive species to various metabolic activities such as vasorelaxation,

macrophage-mediated cytotoxicity, activation inhibition, platelet adhesion and aggregation, regulation of basal and blood pressure. Studies have shown that proteins or specific nutrient deficiency leads to the reduction of nitric oxide [41, 42]. Our study showed that the malnourished animals had decreased nitric oxide production. This result may be due to the lower availability of arginine, basic substrate for the production of nitric oxide [43] or changes in iNOS enzyme system [44]. In animals treated with high-fat diet, we found increased production of nitric oxide, as the largest macrophage activation resulting from stimuli such as MCP-1 [33, 45].

Hydrogen peroxide (H_2O_2) is a key reactive oxygen species in the lysis of phagocytized microorganisms which oxidize the cell membrane to form disulfide bridges between the cysteine of different structural proteins of the bacterium, leading to the death of the same [46]. In our study, we found a lower production of hydrogen peroxide in cultures of 2 h of peritoneal macrophages of malnourished animals [40]. By stimulating macrophages in culture with PMA (*phorbol-myristate-acetate*), we note that the malnourished group was the only one not to respond to the stimulus. This may be a result of protein deficiency that causes changes in the enzyme system that triggers oxidative stress through NADPH oxidase [44].

Table 5 Hydrogen peroxide and nitric oxide production by peritoneal macrophages

	Control	Malnourished	High fat	Control + PMA	Malnourished + PMA	High fat + PMA
Hydrogen peroxide ($\mu\text{M}/2 \times 10^5$ cell)	11.1 ± 0.1	10.2 ± 0.2	11.9 ± 0.2	17.4 ± 0.3	11.2 ± 0.1^b	19.7 ± 0.3^a
	Control	Malnourished	High fat	Control + LPS	Malnourished + LPS	High fat + LPS
Nitric oxide ($\mu\text{M}/\text{mL}$)	109 ± 5	115 ± 5	113 ± 5	248 ± 14	132 ± 6^b	272 ± 30^a

Results represent means \pm SD of the production of hydrogen peroxide and nitric oxide by peritoneal exudate macrophages of control, malnourished and high-fat animals ($n = 18/\text{group}$)

^aIndicates when there was significant difference between the high-fat and control groups ($p < 0.05$)

^bIndicates when there was significant difference between the malnourished and control groups ($p < 0.05$)

Regarding high-fat animals, we found a greater production of hydrogen peroxide (after 2 h) in macrophage culture supernatant, which may be explained by the increased activation of macrophages by pro-inflammatory cytokines that stimulate phagocytic activity, bactericidal, and fungicidal, leading to an increase of reactive oxygen species [47].

The data obtained throughout the study and the realization of this work showed malnourished animals with compromised immune response. The animals submitted to high-fat diet showed increased macrophage activity with inflammation similar to metabolic syndrome. These data are consistent with the literature.

Conclusions

The protein malnutrition impairs the innate immune response by decreasing the amount and functionality of macrophages, which are poorly responding to the activation and production of substances. In the murine model of high-fat diet, we observed an increase in visceral adipose tissue and the presence of an inflammatory process with increasing activity of macrophages in that tissue and systemic changes similar to the metabolic syndrome.

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Authors' contributions

EWS developed the project, performed the experiments, and drafted the manuscript. DCO assisted in the experiments with peritoneal macrophages. AH assisted in the management of mice and obtaining biological samples. JSOB assisted in the management of mice and obtaining biological samples. MMR developed the used diets. RAF helped develop the work design and methods. PB developed the project, guided the actions, and helped in drafting the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

- Monteiro CA, Levy RB, Claro RM, DE Castro IRR, Cannon G. Increasing consumption of ultra-processed foods and likely impact on human health: evidence from Brazil. *Public Health Nutr*. 2011;14(1):5–13.
- FAO (Food and Agriculture Organization). La subnutrición en el mundo en 2010. El estado de la inseguridad alimentaria en el mundo. Roma, 2010. <http://www.fao.org/3/a-i4646s.pdf>. Accessed 16 Oct 2015.
- WHO (World Health Organization). <http://www.who.int/nutgrowthdb/estimates2014/en/>. 2010. Accessed 16 Oct 2015.
- Cuppari L, Coord. Nutrição: nas doenças crônica não-transmissíveis. Barueri: Manole; 2009. p. 512.
- Popkin BM, Adair LS, Ng SW. Global nutrition transition and the pandemic of obesity in developing countries. *Nutrition Reviews*. 2012;70(1):3–21.
- Borelli P, Blatt S, Pereira J, Maurino BB, Tsujita M, Souza AC, Xavier JG, Fock RA. Reduction of erythroid progenitors in protein energy malnutrition. *Brit J Nutr*. 2007;97:307–14.
- Vituri CL, Borelli P, Alvarez-Silva M, Tretin AZ. Alteration of the bone marrow in extracellular matrix in mice undernourished. *Braz J Med Biol Res*. 2001;33:889–95.
- Borelli P, Barros FEV, Nakajima K, Blatt SL, Beutler B, Pereira J, Tsujita M, Favero GM, Fock RA. Protein-energy malnutrition halts hemopoietic progenitor cells in the G0/G1 cell cycle stage, thereby altering cell production rates. *Braz J Med Biol Res*. 2009;42(6):523–30.
- Borelli P, Blatt SL, Rogero MM, Fock RA. Haematological alterations in protein malnutrition. *Rev Bras Hematol Hemoter*. 2004;26(1):49–56.
- Fock RA, Vinolo MAR, Sá Rocha VM, Sá Rocha LC, Borelli P. Protein-energy malnutrition decreases the expression of TLR-4/MD-2 and CD14 receptors in peritoneal macrophages and reduces the synthesis of TNF- α in response to lipopolysaccharide (LPS) in mice. *Cytokine*. 2007;40:105–14.
- Chandra RK. Nutrition and the immune system: an introduction. *Am J Clin Nutr*. 1997;66(2):460–3.
- Souza IP, Kang HC, Nardinelli L, Borelli P. Desnutrição protéica: efeito sobre o espiamento, fagocitose e atividade fungicida de macrófagos peritonais. *Rev Bras Cienc Farm*. 2001;37(2):143–51.
- Schaffner A, Scholmerich J, Salzberger B. Adipose tissue as an immunological organ: toll-like receptors, C1q/TNFs and CTRPs. *Trends Immunol*. 2007;28:393–9.
- Patel PS, Buras ED, Balasubramanyam A. The role of the immune system in obesity and insulin resistance. *J Obes*. 2013;2013(616193):1–9.
- Silveira MR, Frollini AB, Verlengia R, Cavaglieri CR. Correlação entre obesidade, adipocinas e sistema imunológico. *Rev Bras Cineant Desemp Hum*. 2009;11(4):466–72.
- Ramalho R, Guimaraes C. Papel do tecido adiposo e dos macrófagos no estado de inflamação crônica associada a obesidade. *Acta Med Port*. 2008;21:489–96.
- Saaman MC. The macrophage at the intersection of immunity and metabolism in obesity. *Diabetol Metabolic Syndr*. 2011;3:29.
- Zeyda M, Stulnig TM. Adipose tissue macrophages. *Immunol Lett*. 2007;112(2):61–7.
- Ghanin H, Aljada A, Hofmeyer D, Syed T, Mohanty P, Dandona P. Circulating mononuclear cells in the obese are in a pro-inflammatory state. *Circulation*. 2004;110:1564–71.
- Ogawa M. Differentiation and proliferation of hematopoietic stem cells. *Blood*. 1993;84:2844–53.
- Smith CMD. Hematopoietic stem cells and hematopoiesis. *Cancer Control*. 2003;10(1):9–16.
- Oliveira DC, Hastreiter AA, Mello AS, Beltran JSO, Santos EW, Borelli P, Fock RA. The effects of protein malnutrition on the TNF- α and NF- κ B expression via the TNF- α signaling pathway. *Cytokine*. 2014;69(2):218–25.
- Borges MC, Vinolo MA, Crisma AR, Fock RA, Borelli P, Tirapegui J, Curi R, Rogero MM. High-fat diet blunts activation of the nuclear factor- κ B signaling pathway in lipopolysaccharide-stimulated peritoneal macrophages of Wistar rats. *Nutrition*. 2013;29(2):443–9.
- Reeves PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc Writing. *J Nutr*. 1993;1:1939–51.
- Santos EW, Oliveira DC, Hastreiter A, Beltran JS, Silva G, Fock RA, Borelli P. Effects of low protein or high fat diets in hematological and immunological parameters in murine model. *Braz J Pharm Sci*. 2013;49(1):10.
- Rogers P, Webb GP. Estimation of body fat in normal and obese mice. *Br J Nutr*. 1980;43:83–6.
- Herscovitz HB, Holden HT, Belantl JA, Ghaffar A. Manual of macrophage methodology: collection, characterization, and function. M. Dekker: The University of Michigan; 1981. p. 531.
- Griess P. Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt "Ueber einige azoverbindungen." *Chem. Ber*. 1879;12:426–8.
- Pick E, Mizel D. Rapid microassays for measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J Immunol Meth*. 1981; 46:211–26.
- Picardi PK, Calegari VC, Prada PD, Moraes JC, Araujo E, Marcondes CCG, et al. Reduction of hypothalamic protein tyrosine phosphatase improves insulin and leptin resistance in diet-induced obese rats. *Endocrinology*. 2008;149(8):3870–80.

31. Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE, et al. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. *J Neurosci*. 2009;29(2):359–70.
32. Silva G, Tsujita M, Santos EW, Beltran J, Borelli P. Role of AKT/mTOR pathway in fibronectin expression mediated by protein malnutrition. *Exp Hematol*. 2014;42(8):60.
33. Milner JJ, Beck MA. The impact of obesity on the immune response to infection. *Proc Nutr Soc*. 2012;71:298–306.
34. Marcos A, Nova E, Monteiro CA. Changes in the immune system are conditioned by nutrition. *Eur J Clin Nutr*. 2003;57(1):66–9.
35. Crisma AR. Avaliação da hemopoese e da resposta imune inata mediada por macrófagos em camundongos submetidos à recuperação nutricional após desnutrição protéica. 2010. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas. São Paulo: Universidade de São Paulo; 2010.
36. Greenberg S, Grinstein S. Phagocytosis and innate immunity. *Curr Opin Immunol*. 2002;14(1):136–45.
37. Flannagan RS, Harrison RE, Yip CM, Jaqaman K, Grinstein S. Dynamic macrophage “probing” is required for the efficient capture of phagocytic targets. *J Cell Biol*. 2010;191(6):1205–18.
38. May RC, Machesky LM. Phagocytosis and the actin cytoskeleton. *J Cell Sci*. 2001;114(6):1061–77.
39. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*. 1999;17:593–623.
40. Borelli P, Nardinelli L. Protein calorie-malnutrition a decreased in the macrophage's respiratory burst capacity. *Rev Bras Cienc Farm*. 2001;37:51–60.
41. Redmond HP, Gallagher HJ, Shou J, Daly JM. Antigen presentation in protein-energy malnutrition. *Cell Immunol*. 1995;163(1):80–7.
42. Anstead GM, Chandrasekar B, Zhang Q, Melby PC. Multinutrient undernutrition dysregulates the resident macrophage pro-inflammatory cytokine network, nuclear factor-kappaB activation, and nitric oxide production. *J Leukoc Biol*. 2003;74(6):982–91.
43. Cerqueira NF, Yoshida WB. Óxido nítrico: revisão. *Acta Cir Bras*. 2002;17(6):417–23.
44. Petri A, Weitnauer C, Gorch A. Receptor activation of NADPH oxidases. *Antioxid Redox Signal*. 2010;13(4):467–87.
45. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest*. 2006;116(6):1494–505.
46. Almeida ET, Mauro AE, Santana A, Godoy Netto AV, Carlos IZ. Emprego de compostos organometálicos mononucleares de paládio na ativação de macrófagos peritoneais de camundongos. *Química Nova*. 2005;28(3):405–8.
47. Matsuda M, Shimomura I. Increased oxidative stress in obesity: implications for metabolic syndrome, diabetes, hypertension, dyslipidemia, atherosclerosis and cancer. *Obes Res Clin Pract*. 2013;7(5):330–41.

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